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Differential and overlapping expression domains of *Dlx*-2 and *Dlx*-3 suggest distinct roles for *Distal-less* homeobox genes in craniofacial development

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Abstract

During the development of the vertebrate head, cranial neural crest cells migrate into the branchial arches to form many of the structures of the facial skeleton. These cells follow defined developmental pathways and their fates are determined early. We have isolated and characterized the murine *Distal-less* homeobox gene *Dlx*-3 and have performed a comparative analysis of *Dlx*-3 and *Dlx*-2 expression during craniofacial development. In contrast to *Dlx*-2 and other vertebrate *Distal-less* genes, *Dlx*-3 is not expressed in the central nervous system and is expressed in a highly restricted region of the branchial arches. *Dlx*-2 and -3 display temporal and spatial differences in expression in the arches and their derivatives. In later development, these two genes are expressed in both complementary and partially overlapping domains in regions whose development is dependent on epithelial-mesenchymal interactions, such as the developing middle and inner ear, teeth and whisker follicles. The differential expression of *Dlx* genes in the branchial region suggests that they play key roles in craniofacial patterning and morphogenesis.

Keywords: *Distal-less* (*Dlx*); Homeobox genes; Branchial arches; Neural crest; Tooth development; Ear development; Conserved gene families; Craniofacial development

1. Introduction

The vertebrate head is composed of tissues of different embryonic origins with most of the facial skeleton formed from neural-crest-derived mesectoderm. During closure of the neural tube, the cranial neural crest cells migrate ventrally from the neural folds into the frontonasal process and the branchial arches to generate the skeleton, connective tissue and dermis of the face, parts of the skull and the cranial nerves. The migration patterns and fates of cranial neural crest cells have been studied extensively using radiolabeled orthotopic grafts (Noden, 1973, 1975), quail-chick interspecific chimeras (Le Lièvre and Le Douarin, 1975) and vital dye DiI tracing (Lumsden et al, 1991; Serbedzija et al, 1992; Sechrist et al, 1993). From these studies, the migratory pathways of cranial neural crest cells and the precise origin of their different derivatives could be assigned. These findings imply that, during this phase of extensive migration and morphogenesis, a genetic mechanism is required to coordinate the formation and development of the head.

Transplantation experiments have also shown that the fate of the cranial crest cells is predetermined before migration (Lumsden, 1988). For example, first arch crest cells grafted into the area of the second arch produce supernumerary first arch derivatives (Noden, 1983). The basis of this intrinsic pattern-forming potential is not clearly understood. However, the expression of some homeobox genes reflects this early commitment of cells. Most notable among these are the *Hox* genes, which are organized in four paralogous gene clusters and are expressed in a spatially restricted manner along the

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rostrocaudal axis according to their position in the cluster (reviewed in McGinnis and Krumlauf, 1992). In the hindbrain, the anterior borders of expression of specific paralogous *Hox* genes coincide with rhombomere borders. These same genes are expressed in the neural crest cells emigrating from the rhombomeres and ultimately in the branchial arch mesenchyme derived from these cells. The characteristic expression pattern of the *Hox* genes is maintained when the neuroectoderm of specific rhombomeres is transplanted to ectopic sites in the rhombencephalon (Guthrie et al, 1992; Kuratani and Eichele, 1993). That the *Hox* genes are involved in the patterning of the branchial arches is further supported by experiments that altered their expression patterns during mouse development. The inactivation of *Hox* genes normally expressed in the cranial neural crest resulted in profound disturbances of the branchial region, including apparent homeotic transformations (Chisaka and Capecchi, 1991; Lufkin et al, 1991; Chisaka et al, 1992; Gendron-Maguire et al, 1993; Rijli et al, 1993). Similarly, ectopic expression of *Hox* gene products in transgenic mice (Balling et al, 1989) and treatment of developing embryos with retinoic acid (which alters *Hox* gene expression domains) (Kessel and Gruss, 1991; Conlon and Rossant, 1992) elicited craniofacial malformations. However, with the exception of *Hoxa*-2, which is expressed in the neuroepithelium of rhombomere 2 but not in the neural crest derived from it (Prince and Lumsden, 1994), the anteriormost domains of *Hox* gene expression only extend into rhombomere 3 and the second branchial arch (Krumlauf, 1993). *Hox* expression is not detected in the most rostral region of the central nervous system (CNS), including the forebrain, midbrain and associated neural crest, indicating that other genes must be required for the patterning of these anterior regions.

We have previously identified several members of the murine *Distal-less* (*Dlx*) homeobox gene family that encode homeodomains homologous to that of the *Drosophila Distal-less* (*Dll*) homeobox gene (Robinson et al, 1991). The *Dll* gene product is required for the development of the sensory organs in the larva and the distal components of adult appendages (Sunkel and Whittle, 1987; Cohen and Jürgens, 1989). It is now apparent that the *Distal-less* gene family is both large and extremely conserved. Family members have been identified from several vertebrate species (Price et al, 1991; Robinson et al, 1991; Porteus et al, 1991; Asano et al, 1992; Beauchemin and Savard, 1992; Ekker et al, 1992; Dirksen et al, 1993, 1994; Papalopulu and Kintner, 1993; Selski et al, 1993; Simeone et al, 1994). A major site of expression of the previously characterized murine *Dlx* genes, *Dlx*-1, -2, -5 and-6, is the ventral forebrain, suggesting their involvement in the regionalization of this part of the CNS (Price et al, 1991; Porteus et al, 1991; Robinson et al, 1991; Simeone et al, 1994). In this paper, the molecular characterization and expression pattern of another member of this gene family, *Dlx*-3, is described and compared to *Dlx*-2. In contrast to *Dlx*-2, *Dlx*-3 is not expressed in the CNS. However, both *Dlx*-2 and *Dlx*-3 are differentially expressed, both spatially and temporally, in the branchial arches and facial region during development and in the developing teeth and whiskers. Each gene has a distinct expression pattern in the vestibular portion of the inner ear and in the middle ear. Their restricted and temporally regulated expression, first in the migrating cranial neural crest and subsequently in structures that are derived from the neural crest, suggests that the *Dlx* genes may participate in the patterning and morphogenesis of the craniofacial region.

2. Results

2.1. Isolation and characterization of Dlx-3 cDNA sequences

In an earlier study, we identified several members of the murine *Distal-less* (*Dlx*) homeobox gene family (*Dlx*-1, -2, -3 and -4) using polymerase chain reaction (PCR) based methods, and described the expression pattern of the *Dlx*-2 gene in the forebrain (Robinson et al, 1991). In order to isolate cDNA sequences corresponding to the other murine *Dlx* genes, we screened several embryonic cDNA libraries with a mixture of the PCR amplified *Distal-less* homeobox sequences. Two cDNA clones containing the homeobox sequence designated *Dlx*-3 were isolated. We were unable to isolate cDNA clones containing the *Dlx*-4 homeobox sequence. Since the nucleotide sequence of the amplified *Dlx*-4 homeobox is nearly identical to that of *Dlx*-3 and since it was only found once among amplified gene products, it is possible that this sequence was a PCR-generated variant of *Dlx*-3.

The two partially overlapping *Dlx*-3 cDNA clones, containing inserts of 1.1 and 0.6 kb, were not full-length and lacked an initiating methionine codon. A genomic DNA clone containing more 5′ sequence was isolated that, together with the cDNA clones, allowed the entire amino acid sequence to be predicted (Fig. 1). This sequence contained a methionine codon preceded by a consensus sequence for initiation of translation (Kozak, 1989) and was very similar to the related *Xenopus* (Dirksen et al, 1994) and zebrafish (Ekker et al, 1992) genes (see Fig. 2).

The predicted *Dlx*-3 protein contains 287 amino acids. The homeodomain is located in the central portion of the protein as it is in the predicted *Dlx*-2 protein (Robinson et al, 1991) and in that of the distantly related homeobox gene *Msx*-1 (formerly *Hox* 7.1) (Hill et al, 1989). Recently, other vertebrate *Dlx*-3-related genes were isolated from the newt (Beauchemin and Savard, 1992), zebrafish (Ekker et al, 1992) and frog

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   1  ATGAGCGGCTCCTTCGATCGCAAGCTCAGCAGCATCCTCACCGACATCTCCAGCTCGCTC   60
   1  M  S  G  S  F  D  R  K  L  S  S  I  L  T  D  I  S  S  S  L    20
  61  AGCTGCCATGCGGGCTCCAAGGACTCGCCCACCCTGCCCGAATCTACAGTCACTGACCTG  120
  21  S  C  H  A  G  S  K  D  S  P  T  L  P  E  S  T  V  T  D  L    40
 121  GGCTATTACAGCGCTCCTCAGCATGACTACTACTCGGGCCAGCCCTACGGCCAGACGGTG  180
  41  G  Y  Y  S  A  P  Q  H  D  Y  Y  S  G  Q  P  Y  G  Q  T  V    60
 181  AACCCCTACACCTACCACCACCAGTTCAATCTCAATGGGCTCGCAGGCACCGGCGCTTAC  240
  61  N  P  Y  T  Y  H  H  Q  F  N  L  N  G  L  A  G  T  G  A  Y    80
 241  TCGCCCAAGTCGGAATATACCTACGGGGGATCCTATAGGCAGTACGGAGCGTACCGGGAG  300
  81  S  P  K  S  E  Y  T  Y  G  G  S  Y  R  Q  Y  G  A  Y  R  E   100
 301  CAGCCTTTGCCTGCCCAGGACCCAGTGTCGGTGAAAGAGGAGCCGGAAGCCGAGGTTCGC  360
 101  Q  P  L  P  A  Q  D  P  V  S  V  K  E  E  P  E  A  E  V  R   120
 361  ATGGTGAACGGCAAGCCCAAAAAGGTCCGAAAGCCGCGAACGATCTACTCCAGCTATCAG  420
 121  M  V  N  G  K  P  K  K  V  R  K  P  R  T  I  Y  S  S  Y  Q   140
 421  CTGGCTGCCCTGCAGCGCCGTTTCCAGAAAGCCCAGTATCTGGCCTTGCCTGAGCGCGCC  480
 141  L  A  A  L  Q  R  R  F  Q  K  A  Q  Y  L  A  L  P  E  R  A   160
 481  GAGCTAGCTGCACAGCTGGGCCTCACACAAACACAGGTGAAAATCTGGTTCCAGAACCGC  540
 161  E  L  A  A  Q  L  G  L  T  Q  T  Q  V  K  I  W  F  Q  N  R   180
 541  CGCTCCAAGTTCAAAAAGCTCTATAAGAATGGGGAGGTGCCGCTGGAACACAGCCCCAAC  600
 181  R  S  K  F  K  K  L  Y  K  N  G  E  V  P  L  E  H  S  P  N   200
 601  AACAGTGACTCCATGGCCTGCAACTCACCGCCGTCACCAGCACTCTGGGACACATCTTCC  660
 201  N  S  D  S  M  A  C  N  S  P  P  S  P  A  L  W  D  T  S  S   220
 661  CATTCCACGCCAGCCCCTGCCCGCAATCCGCTGCCCCCACCGCTCCCATACAGTGCCTCC  720
 221  H  S  T  P  A  P  A  R  N  P  L  P  P  P  L  P  Y  S  A  S   240
 721  CCCAACTACCTGGACGACCCCACCAACTCCTGGTACCACACACAGAACCTCAGTGGACCC  780
 241  P  N  Y  L  D  D  P  T  N  S  W  Y  H  T  Q  N  L  S  G  P   260
 781  CACTTACAGCAGCAGCCTCCTCAGCCGGCTACCCTGCACCATGCCTCCCCTGGGCCCCCG  840
 261  H  L  Q  Q  Q  P  P  Q  P  A  T  L  H  H  A  S  P  G  P  P   280
 841  CCTAACCCTGGGGCTGTGTACTGAGTACCCACCTGGCCTGCGCCCCTCCACGAAGGACCC  900
 281  P  N  P  G  A  V  Y  *                                        300
 901  CCTCCAGGACCAGGCAGAAGGTGCCCTGTCCTAGCGACACTCAGGAATCATTGAGGGGCA  960
 961  CAGGGGGAAAGACTCCCTTCCCTCTCCCTTGTCCCTTCTTCCAGGGGCCCAACAACCTCC 1020
1021  AGATGACAAATGCATGGACCGAGGATGCCCCCCAATCTCCCTCCCCTTGCTTAGACTGGG 1080
1081  GTGCCCTCCAGACGGCGAGGAGTTCTACCCCAGTGGGGACAGCACATGCTCTCTGCTCCA 1140
1141  GGAACCCGGATTGCCTCTAGATGGCTCATCACTTTCCAGCTTTTCAAACACAGTAGAGAC 1200
1201  CTCCAAAATGGGAGCCAGAGTGTTTGCAGGTCCACCTGTGCTGGGGCACCAGGCGCCACG 1260
1261  GATTCCAGCACAGCCAGACCTAAAGCACCAAGCCG  1295
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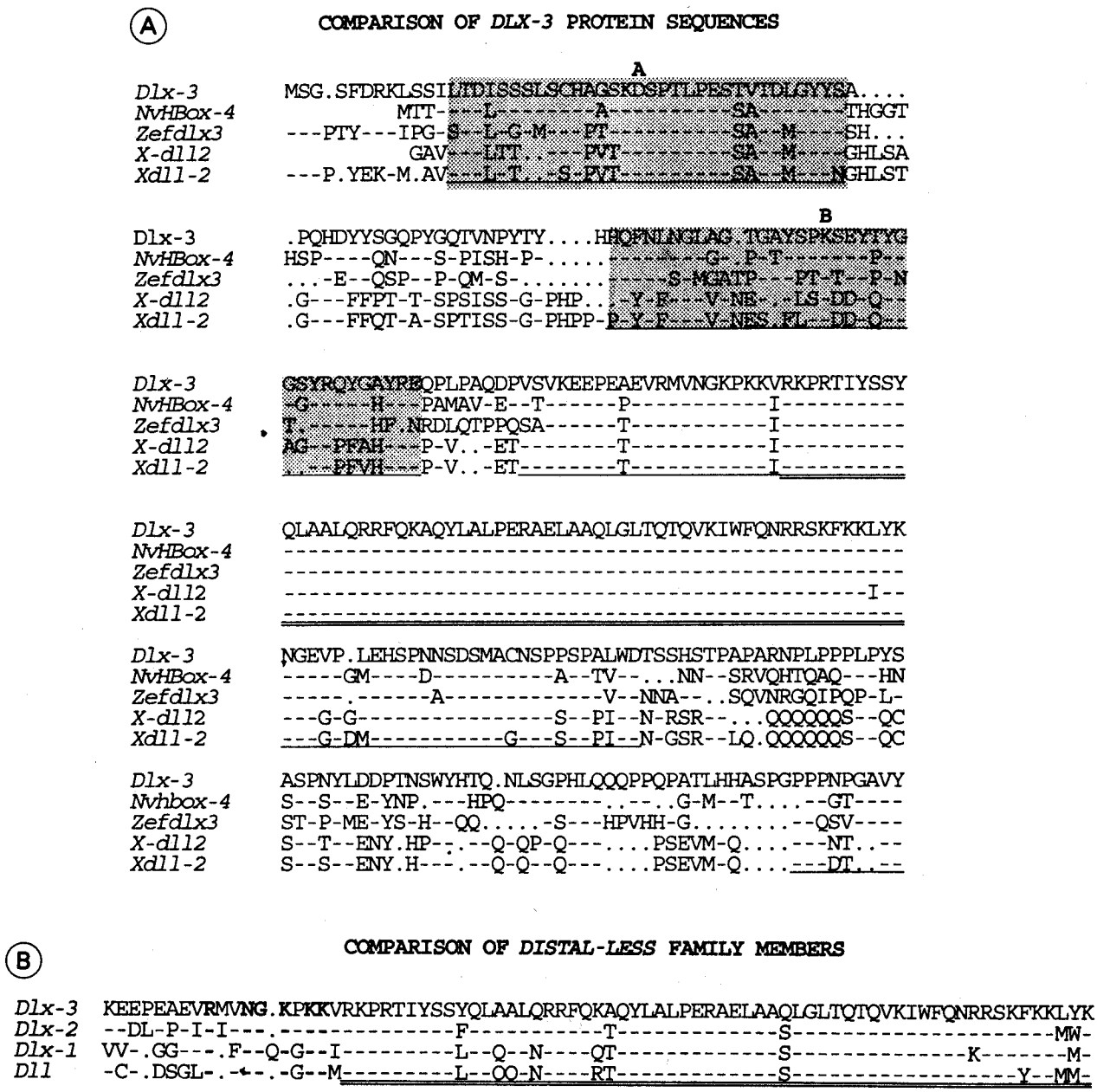
Fig. 1. cDNA sequence and deduced amino acid sequence of *Dlx*-3. The homeobox sequence is underlined.

(Papalopulu and Kintner, 1993; Dirksen et al. 1994). A comparison of these sequences is shown in Fig. 2A. All of the genes display a high degree of conservation over the whole length of the protein and may, in fact, be true orthologs (the *Xenopus* genes may represent the two pseudo-allelic genes in this tetraploid organism). In all five genes, 46% of the amino acids are identical and another 9% of the sequence is shared by four of the five proteins. An unusually long 'extended homeodomain' is found, comprised of 21 residues upstream and 29 downstream of the classical helix-loop-helix homeodomain (Beauchemin and Savard, 1992). Of these 110 amino acids, 89% are identical and 93% are conserved in four of the five sequences. It is also noteworthy that the block of conserved residues preceding the homeodomain has a high concentration of charged residues (nine out of 20) (Fig. 2B) and has the potential to form α-helical structures (Devereux et al, 1984).

A comparison of the predicted protein sequence of all known members of the murine *Dlx* gene family (*Dlx*-1,


A
COMPARISON OF DLX-3 PROTEIN SEQUENCES
A
B
B
COMPARISON OF DISTAL-LESS FAMILY MEMBERS
C
DISTAL-LESS DOMAIN
Dlx-3
NvHBox-4
Zefdlx3
X-dll2
Xdll-2
Dlx-2
X-dll4 (XDLL1)
X-dll3
Consensus
Nvhbox-4
Dlx-1
Dll

-2 and-3) (Price et al, 1991; Porteus et al, 1991; Robinson et al, 1991) and the *Drosophila Distal-less* gene (Vachon et al, 1992) shows that the homology upstream of the homeodomain is also conserved among the fly gene and the different mouse *Distal-less* family members (Fig. 2B). In this stretch of 18 amino acids, six residues are identical. However, the similarity between the *Drosophila* and the mouse sequences drops outside of the homeodomain. Although only a partial protein sequence is available for *Dlx*-1 (Price et al, 1991), it is obvious that *Dlx*-2 and *Dlx*-3 are more similar to each other than *Dlx*-1 (data not shown). Only sequence from the homeodomain is available for the *Dlx*-5 and -6 genes (Simeone et al, 1994), precluding comparison.

Two other conserved blocks with 56 and 24% identity, block A and B, respectively, are located upstream of the homeodomain of all *Dlx*-3 genes (Fig. 2A and C). The last nine C-terminal amino acids are conserved as well. Block A near the N-terminus represents a '*Distal-less* domain' that is highly conserved in all of the vertebrate *Distal-less* genes, but is not found in the *Drosophila* gene (Fig. 2C). The N-terminal residues of block A appear to be characteristic for orthologous (or subfamily) members, whereas the C-terminal portion is highly conserved in all the genes.

2.2. Analysis of Dlx-3 expression

By Northern blot analysis with *Dlx*-3 probes, a 2.5-kb transcript was detected in the RNAs of embryos of 9 days post coitum (p.c.) and older (data not shown). Hybridization to RNA samples from microdissected embryos showed that this transcript was expressed in the skin and in the snout (Fig. 3). In contrast to the other well-characterized murine *Dlx* genes, *Dlx*-1, -2, -5 and-6, *Dlx*-3 was not detectably expressed in the CNS. In adult tissues, *Dlx*-3 was expressed only in skin but not in brain, liver, spleen, lung, kidney, salivary gland and skeletal muscle (data not shown). The differential hybridization of these *Dlx* probes confirmed that, under the conditions used, the probe for *Dlx*-3 did not cross-hybridize with other *Dlx* gene transcripts which are known to be strongly expressed in the developing brain (Price et al, 1991; Robinson et al, 1991; Porteus et al, 1991; Simeone et al, 1994).

2.3. In situ localization of Dlx gene transcripts: Expression in the branchial arches

A detailed comparative analysis of the temporal and spatial expression pattern of *Dlx*-2 and-3 in the developing head was performed (Figs. 4–8). Early expression domains were visualized by whole mount in situ hybridization of intact embryos (8–10.5 days p.c.) and isolated heads from 12.5-day-p.c. embryos. As has been reported for *Dlx*-1 (Dollé et al, 1992) and *Dlx*-2 (Bulfone et al, 1993), a major site of expression of *Dlx*-3 was in the branchial arches. Expression of *Dlx*-2 precedes that of *Dlx*-3 and was initially detected in day 8.5 embryos in two bands of cells extending from the hindbrain into the branchial arches (Fig. 4A). The appearance of the labeled cells was consistent with that of migrating neural crest cells. By 9.5 days p.c., after the neural crest cells have completed emigration, the *Dlx*-2 expressing cells were located in the maxillary and mandibular components of the first arch and in the second arch (Fig. 4B). At this stage, *Dlx*-3 expression was first detected in the distal tips of the arches (Fig. 4C) where post-

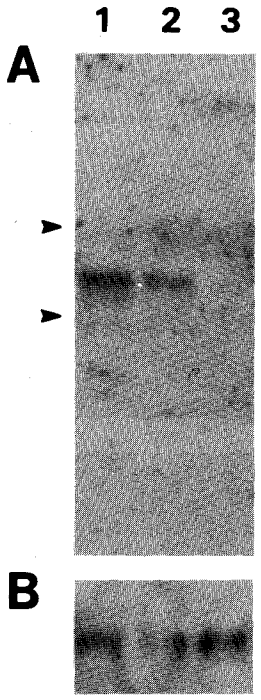


Fig. 3. Detection of *Dlx*-3 by Northern blot analysis. Total RNA (20 μg) from microdissected tissues of 16.5 day p.c. embryos was separated on a 1.2% formaldehyde-agarose gel, blotted and hybridized with *Dlx*-3 (A) and GAPDH (B) probes. Lane 1: dorsal skin, lane 2: snout, lane 3: brain. Arrows indicate positions of 28S and 18S ribosomal RNA bands.

Fig. 2. Comparison of vertebrate *Dlx*-3 protein sequences. (A) The amino acid sequences of Dlx-3 from the mouse, newt, zebrafish and frog are aligned. The two conserved domains in the N terminal portion (Box A and Box B) are indicated by the boxes. The conserved regions flanking the homeodomain are underlined and the homeodomain is double underlined. (B) Comparison of the homeodomain and upstream flanking sequence from *Drosophila* and mouse. The homeodomain is underlined. The conserved amino acids preceding the homeodomain are indicated by asterisks. (C) *Distal-less* domain near the N terminus. This region is conserved among the genes of the vertebrate *Distal-less* family. The amino acids which are identical among orthologs are indicated by asterisks. The sequence at the N terminal part is specific for orthologous genes of different vertebrate organisms, while the C terminal portion is conserved in all the *Distal-less* genes. The N and C terminal regions are separated by a space.

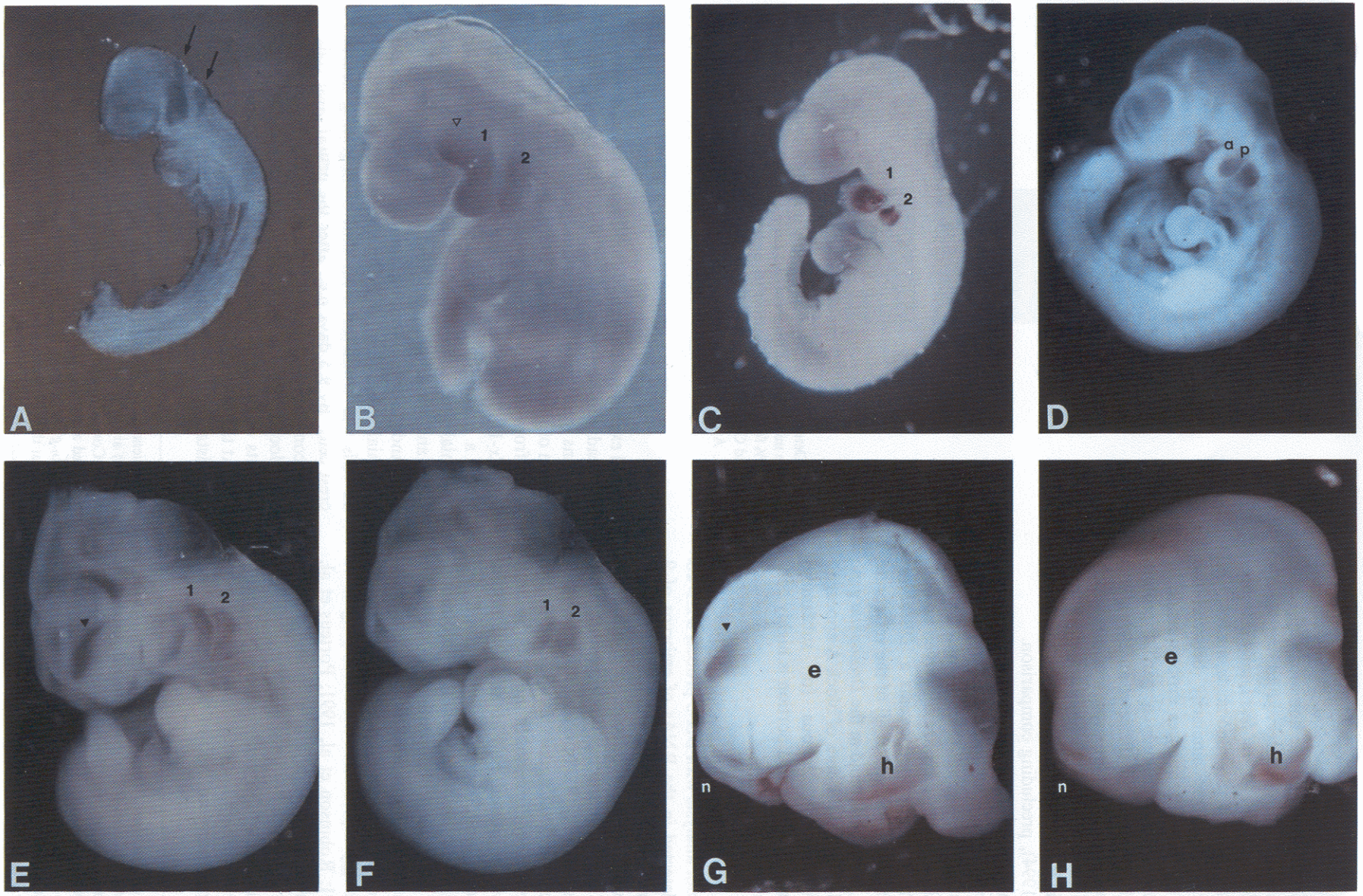


Fig. 4. Whole mount in situ hybridization with *Dlx*-2 and *Dlx*-3 probes. (A) *Dlx*-2 hybridization to an 8.5-day-p.c. embryo. Expression in the branchial arch region extends from the hindbrain (arrows) into the arch. (B) *Dlx*-2 hybridization to a 9.5-day-p.c. embryo. Arrowhead indicates position of *Dlx*-2 hybridization in the maxillary portion of the first arch. (C) *Dlx*-3 expression in a 9.5-day-p.c. embryo in branchial arches 1 and 2. (D) *Dlx*-3 expression in a 10-day-p.c. embryo. Transcripts are present in the posterior (p) but not the anterior (a) portion of the first branchial arch. (E and F) 10.5-day-p.c. embryos. (E.) *Dlx*-2 expression is seen in the branchial arches and forebrain (the basal telencephalon, indicated by the arrow head, and the more caudal ventral diencephalon). (F) *Dlx*-3 transcripts are restricted to the posterior first arch and the second arch. In addition, note that the expression domain of *Dlx*-3 is more restricted to the distal aspect of arches 1 and 2 than that of *Dlx*-2. (G and H) Isolated heads of 12.5-day-p.c. embryos. *Dlx*-2 (G) and *Dlx*-3 (H) are expressed in the auditory hillocks (h). Note the slightly more extensive expression domain of *Dlx*-2 in this region. *Dlx*-2 is also expressed in the ventral olfactory bulbs (arrowhead), the oral epithelium and the external naris (n). 1, 2 indicate first (mandibular) and second (hyoid) branchial arch, respectively. (e) eye.

migratory neural crest cells are located. In slightly older embryos, the expression of *Dlx*-3 became restricted to the caudal portion of the mandibular process (Fig. 4D). As the more posterior arches developed, *Dlx*-2 expression was also evident there (Fig. 4E). Expression of *Dlx*-3 was found in a more limited region in the first two branchial arches only (Fig. 4F). In the head of a 12.5-day-p.c. embryo, *Dlx*-2 and -3 expression was seen only in a small area around the branchial cleft which will form the auditory hillocks (Fig. 4G and H). As in the earlier stages, the expression of *Dlx*-3 was restricted to a smaller cell population than *Dlx*-2 (Fig. 4G and H). At this stage expression of *Dlx*-2 was also found in the oral epithelium and the nostrils as well as in the basal forebrain (Fig. 4G).

The whole mount hybridization method is less effective at later stages of development due to difficulties with penetration of the probe. Therefore, we performed in situ hybridization of radiolabeled probes to sections of embryos to confirm and extend the expression analysis. Sections through the branchial region of up to 11.5 days p.c. showed that the epithelium and the mesenchyme expressed *Dlx*-3 uniformly. In 11.5 day embryos, the anterior border of expression was rather sharp and extended through the middle of the first branchial arch. Internally, the lateral extent of expression ended at the branchial pouch (Fig. 5A and B). Up to day 12.5 p.c., the expression pattern in the branchial region did not change and was seen in the mandibular process and the second branchial arch (Fig. 5C and D). At this time, *Dlx*-3 expression was not restricted to a distinct morphological structure but was mainly found in uniformly dense mesenchymal cells.

2.4. Expression of Dlx during craniofacial development

Starting about day 12.5 of embryonic development, the facial and branchial region undergoes extensive morphological changes as the final shape of the face develops. Condensations form in the mesenchyme which subsequently ossify and give rise to the bones of the skull and the face. The Meckel's cartilage in the mandible becomes clearly visible. At 13.5 days p.c., *Dlx*-3 expression was seen in the area under the first branchial cleft from which the external ear develops (Fig. 5E and F). Presumably this region is derived from *Dlx*-3 expressing cells that, at earlier stages, were positioned more externally at the tip of the anterior second branchial arch and which have shifted internally during facial morphogenesis.

Expression of *Dlx*-3 was also seen in the epithelium of the external naris (Fig. 5G and H). The hybridization signal was restricted to the respiratory epithelium, while no expression was detected in the more internal sensory epithelium. In addition, *Dlx*-3 was expressed in a narrow band of mesenchymal cells that form a ring enclosing the nasal epithelium, which presumably forms the nasal capsule (Fig. 5G and H). Strong hybridization of *Dlx*-3 was also seen in a group of condensed cells in the vicinity of Meckel's cartilage that corresponds to the future ossification center of the mandible (Fig. 7E and F).

Weak *Dlx*-3 expression was found in discrete regions of the mesenchyme between the hindbrain and the oral cavity (Fig. 5I and J). The expression of *Dlx*-3 did not coincide with a single distinct anatomical structure but was associated with loosely organized mesenchyme in the area of the middle ear. During further development, the expression of *Dlx*-3 was progressively reduced such that by 14.5 day p.c., only a few mesenchymal cells in the maxillary region remain labeled (data not shown).

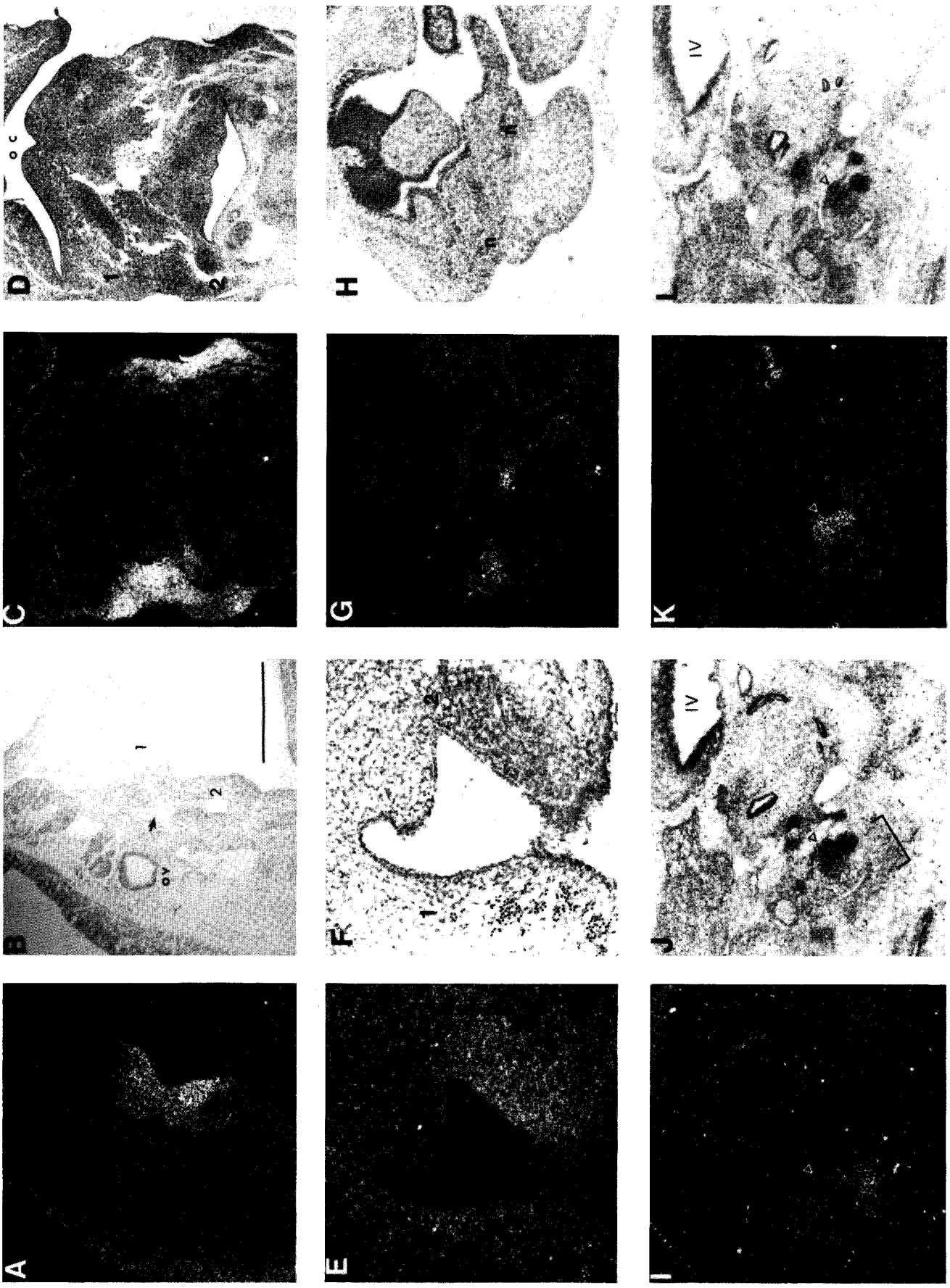
The expression domain of *Dlx*-2 in the facial mesenchyme abutted, and partially overlapped, that of *Dlx*-3 and was restricted to condensed mesenchyme lying close to the tubotympanic recess, a cavity which forms from the first branchial pouch. These mesenchymal cells are the primordia of two first arch-derived middle ear ossicles, the malleus and incus, which originate from the proximal portion of Meckel's cartilage and the quadrate cartilage, respectively (Fig. 5K and L) (Sadler, 1990).

2.5. Dlx expression in the inner ear

The inner ear develops from the otic vesicle which is derived from the invaginating surface ectoderm of the otic placode. During further development, the vesicle divides into the ventrally located saccule and cochlea, where Corti's organ develops, and a dorsal part which gives rise to the membranous labyrinth. The labyrinth is the organ that senses motion equilibrium and consists of the semicircular canals and the utricle (Sher, 1971).

Around day 11.5 p.c., the otic vesicle appears as an ovoid sac whose dorsal walls consist of simple cuboidal epithelium while the ventral walls are formed by pseudostratified epithelia with 2–3 layers of cells. *Dlx*-2 and -3 were expressed at low levels as the otic vesicle begins to elongate and the vestibular labyrinth and cochlear portions become distinguishable. At 12.5 days p.c., the expression of both genes was restricted to the vestibular part (Fig. 6A-C). The pattern of expression appeared discontinuous within an apparently homogeneous cell population. Comparison of a series of adjacent sections revealed that the expression domains of the two genes were partially exclusive.

On 12.5 days p.c., a rapid sequence of morphogenetic events leads to the formation of the semicircular canals which develop as pouches projecting from the wall of the otocyst. The opposed walls of the pouches fuse in the center but remain open along the rim, thus forming the canals (Martin and Swanson, 1993). On day 13.5 p.c., all three semicircular canals are present. At this stage, the restriction of *Dlx* expression to the vestibular region became even more pronounced. Sections through the center of the inner ear at the transition from the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vestibular labyrinth into the saccule showed that expression of *Dlx*-3 was only found in the epithelium of the utricle and the semicircular canals (Fig. 6D and E). Both *Dlx*-2 and *Dlx*-3 were expressed in the semicircular canals (Fig. 6F–I). However, this expression was not uniform as both hybridizing and non-hybridizing regions were noted with both probes. Because of the dynamic growth and bending of the developing ear during this period (Sher, 1971), it was not possible to determine precisely the nature of structures showing differential hybridization. More detailed analysis by reconstruction of serial sections should resolve this issue in the future. The expression declined after 13.5 day p.c. and could not be detected in 14.5 day embryos.

2.6. Dlx expression in the developing teeth

As in the branchial arches, the expression of *Dlx*-2 slightly preceded that of *Dlx*-3 and was slightly stronger than *Dlx*-3 throughout embryonic tooth development. On 12.5 days p.c., *Dlx*-2 was expressed in the oral epithelium and the adjacent mesenchyme in the areas where the teeth were developing (Fig. 7A and B). One day later, as the dental lamina developed into an epithelial bud surrounded by dental mesenchyme, both genes were expressed (Fig. 7C–F). At this stage, the expression of *Dlx*-2 shifted to the buccal portion of the anlage. In both incisors and molars, transcripts were found predominantly in the dental epithelium and the adjacent mesenchyme on the side opposing the cheek (Fig. 7D and H). *Dlx*-3 was expressed at a low level, predominantly in the mesenchyme of the dental papilla (Fig. 7F and I). Subsequently, a cap and bell shaped anlage is formed as the deep surface of the epithelial bud invaginates to partially surround the papillar mesenchyme. The patterns of *Dlx* expression were maintained until the bell stage when final differentiation commences (Fig. 7J–L). As the cells become organized at the epithelial mesenchymal interface, the mesenchymal cells differentiate into odontoblast cells and the cells of the inner enamel epithelium form the ameloblast layer. *Dlx*-2 expression was restricted to the ectoderm-derived ameloblast layer (Fig. 7K), while *Dlx*-3 expression was seen in both the ameloblasts and in the odontoblasts, known to be derived from the cranial neural crest (Lumsden, 1988) (Fig. 7L).

2.7. Dlx Expression in whisker follicles

Development of the whisker follicles starts at day 11.5 in the maxillary process. In an anterior to posterior progression, five rows of placodal thickenings are formed in the epidermis. The underlying mesenchyme condenses and eventually becomes surrounded by the ingrowing epithelial cells to form the dermal papillae. Those epithelial cells adjacent to the papilla, the hair matrix cells, start to divide rapidly and give rise to the more differentiated cells of the hair follicle (Hardy, 1992). At 12.5 days p.c., expression of *Dlx*-2 and -3 was visualized in a patchy pattern in the epithelium of the upper lip (Fig. 8A–C). These regions corresponded to the positions of early whisker follicles. As the follicles developed, the expression of *Dlx*-3 increased strongly, whereas *Dlx*-2 expression was extinguished. In whisker pads from 13.5-day-p.c. embryos, a clear gradient of *Dlx*-3 expression became apparent as the whisker follicles differentiate in an anterior-posterior sequence (data not shown). On day 14.5, all follicles expressed *Dlx*-3 (Fig. 8D, F and G) and the expression remained strong until birth (not shown). A slight decrease of expression was seen in whiskers from 3-day-old mice, the latest time point analyzed in this study. Sections through 15.5-day-p.c. whisker follicles after whole mount hybridization revealed that the expression of *Dlx*-3 was localized to a small population of hair matrix cells adjacent to the dermal papilla (Fig. 8E). Similar labeling was seen in the hair follicles in sections of newborn skin (data not shown).

3. Discussion

3.1. Domains of the vertebrate Distal-less proteins are highly conserved

In this study we present the complete cDNA sequence of the murine *Dlx*-3 gene, originally identified as a PCR-generated fragment (Robinson et al, 1991), and describe its expression pattern during mouse embryogenesis. Comparison of the predicted amino acid sequence of *Dlx*-3 reveals conservation of the sequences immediately preceding and following the homeodomain among *Dlx* family members. This 'extended homeodomain' has also been noted in other homeobox gene families (for example Takahashi and Le Douarin, 1990; reviewed in

Fig. 5. *Dlx* expression in the branchial arches and their derivatives. (A and B) Sagittal section of 11.5-day-p.c. embryo hybridized to *Dlx*-3 antisense probe. Transcripts are detected in the posterior portion of the first (1) and the second (2) branchial arch extending to the branchial pouch (arrow). ov, otic vesicle. (C and D) Coronal section of 12.5-day-p.c. embryo hybridized to *Dlx*-3 antisense probe. Note expression in the lateral region of the first and second arch. (E–L) Sagittal sections of 13.5-day-p.c. embryo. (E and F) *Dlx*-3 transcripts are found in the mesenchyme abutting the first branchial cleft. (G and H) Section through the frontonasal process. *Dlx*-3 is expressed in the nasal capsule (n). (I–L) Adjacent sections through the region of the future middle ear. (I and J) *Dlx*-3 hybridizes to a diffuse expression domain in the mesenchyme (brackets). (K and L) Expression of *Dlx*-2 in the condensed mesenchyme from which the malleus and incus develop (arrowhead). oc, oral cavity; ov, otic vesicle; IV, Fourth ventricle. Dark field (A, C, E, G, I and K) and corresponding bright field (B, D, F, H, J and L). Bar = 500 μm in A–D and I–L; 200 μM in E and F; 300 μm in G and H.

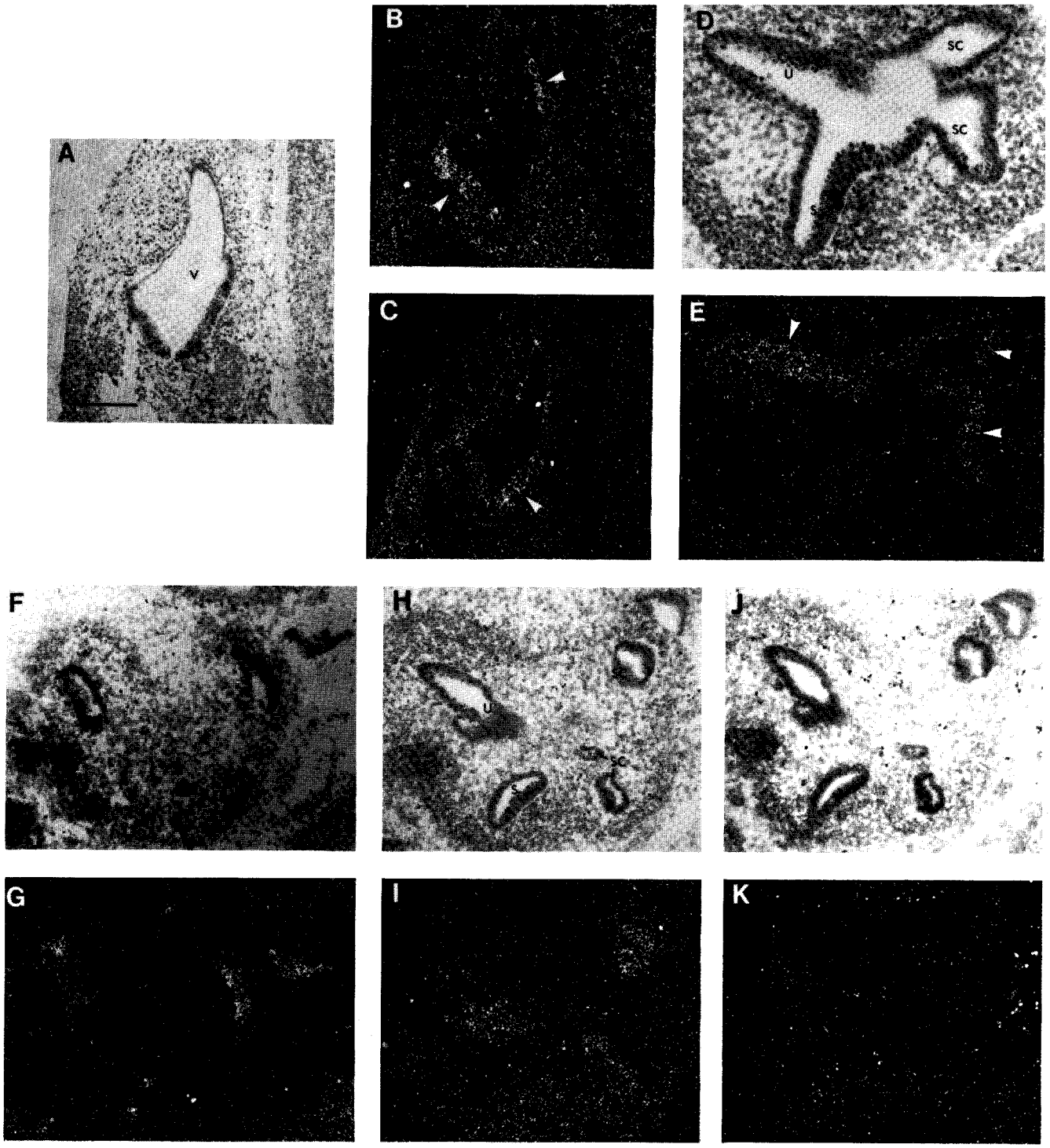

Fig. 6. *Dlx* expression in the inner ear. (A–C) Coronal section through vestibular portion of the otic vesicle of 12.5-day-p.c. embryo. *Dlx*-2 (B) and *Dlx*-3 (C) are expressed in distinct, partially overlapping domains. (D–K) Sagittal sections of 13.5-day-p.c. embryo. (D and E) Hybridization of *Dlx*-3 probe to a section at the transition from the vestibular to the saccular portion. Note presence of *Dlx*-3 transcripts in the epithelium of the utricle (u) and the semicircular canals (sc). No expression is detectable in the saccule (s). (F and G) Expression of *Dlx*-2 in the semicircular canals. (H and I) Expression of *Dlx*-3 is seen in the semicircular canals and the utricle. This section is slightly medial to sections F and G. (J and K) Control hybridization of a neighboring section with *Dlx*-3 sense probe shows only background labeling. Bar = 170 μm in A–C, 100 μm in D and E and 200 μm in F–K.

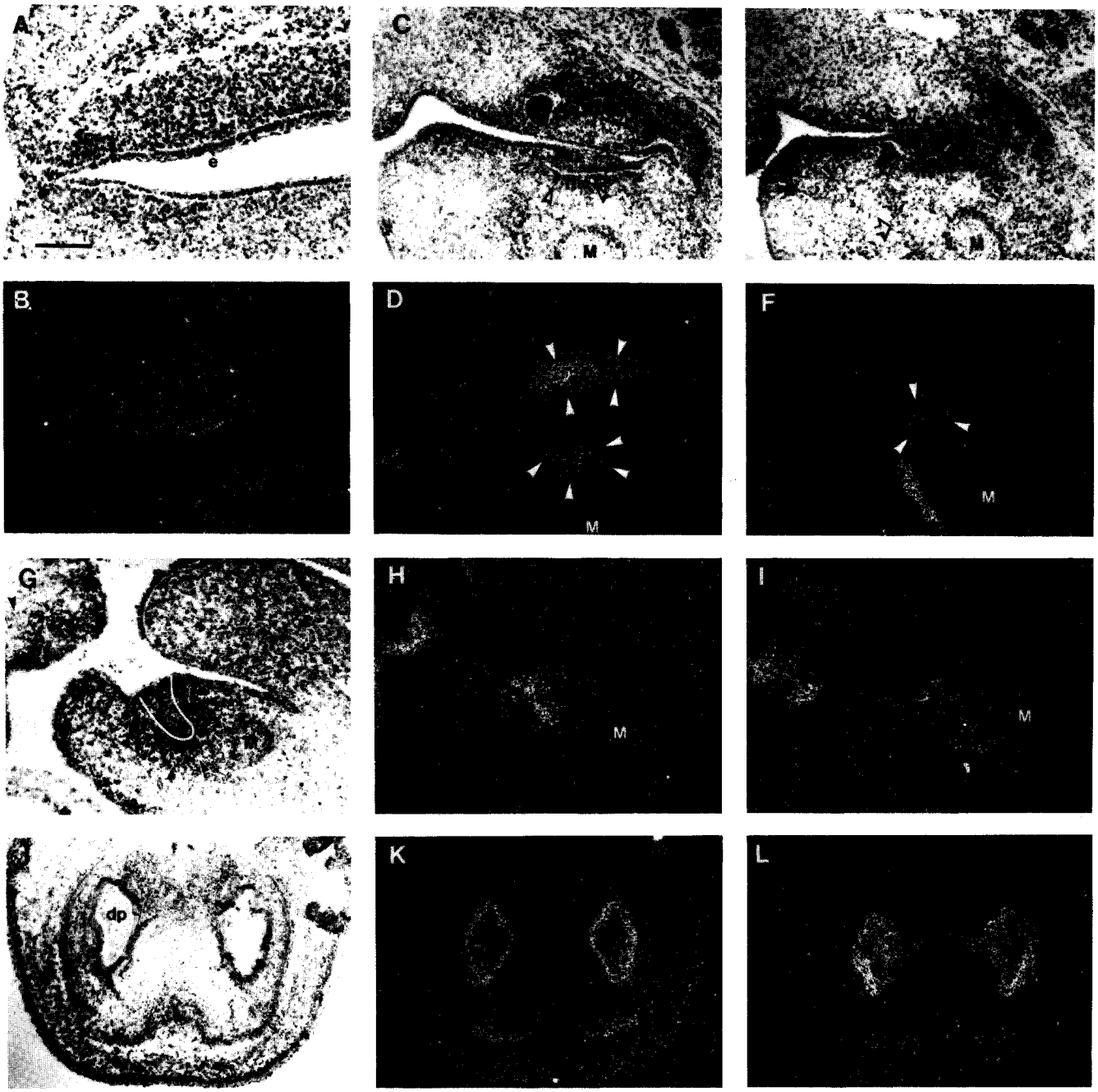

Fig. 7. Expression of *Dlx*-2 and -3 during tooth development. (A and B) Coronal section of 12.5-day-p.c. embryo. *Dlx*-2 expression is seen in the oral epithelium (e) and the adjacent mesenchyme (m) in the areas where teeth begin to develop. (C–F) Sagittal section through the first molars of a 13.5-day-p.c. embryo. (C and D) *Dlx*-2 is expressed in the dental lamina (dl) of the lower molar and in the surrounding mesenchyme (also seen in the upper jaw) (arrowheads). (E and F) Expression of *Dlx*-3 is seen in the dental mesenchyme. The open arrowhead indicates the condensed mesenchyme corresponding to the developing mandible that shows robust expression of *Dlx*-3. (G–H) Sagittal section through incisors of a 13.5-day-p.c. embryo. (H) shows expression of *Dlx*-2 concentrated to the dental epithelium and the adjacent mesenchyme in the buccal portion of the anlagen. (I) Expression of *Dlx*-3 is more diffuse and extends into the follicular mesenchyme. The arrow heads in (G) indicate area of *Dlx*-3 expression. The area of *Dlx*-2 expression is encompassed by a white line. (J–L) Coronal section of 16.5-day-p.c. incisor. (B) *Dlx*-2 expression is restricted to the ameloblast layer. (C) *Dlx*-3 is expressed in the ameloblast layer as well as in the mesenchyme of the dental papilla (dp). M, Meckel's cartilage. Bar = 100 μm in A and B; 200 μm in C–L.

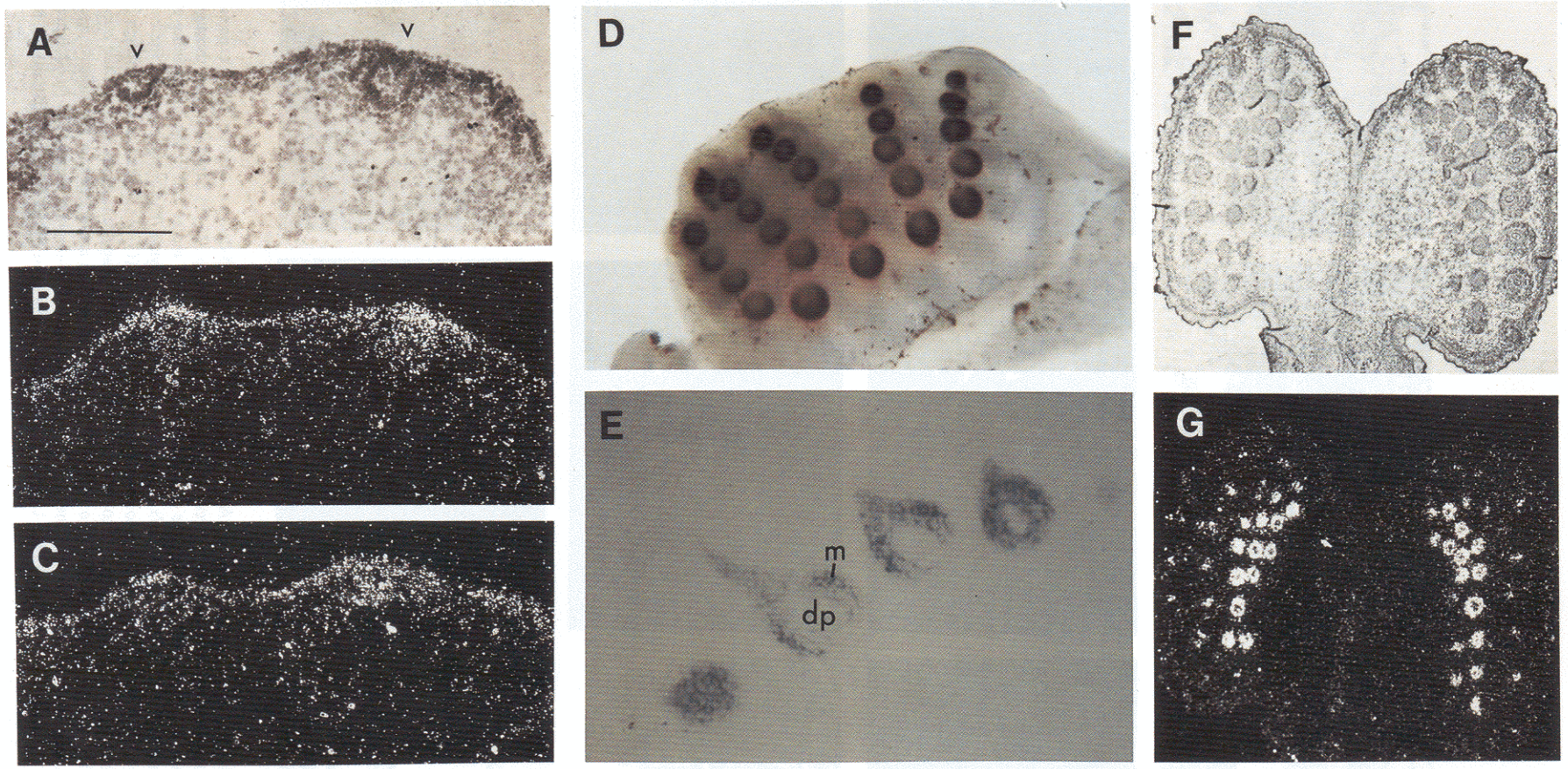

Fig. 8. *Dlx* expression in whisker follicles. (A–C) Sagittal section through 13.5-day-p.c. upper lip. *Dlx*-2 (B) and *Dlx*-3 (C) are expressed in early vibrissa anlagen (v). (D) Whole mount in situ hybridization of *Dlx*-3 probe to 15.5-day-p.c. embryonic whisker pad viewed from underside. (E) Section through whisker pad seen in (D). Expression of *Dlx*-3 is restricted to the matrix cell layer (m) surrounding the dermal papilla (p). (F and G) Coronal section through upper lip of 16.5-day-p.c. embryo. Strong expression of *Dlx*-3 is seen in the matrix cells. Bar = 200 μm in A–C; 170 μm in D and E; 500 μm in F and G.

Krumlauf, 1992). Interestingly, there is also a region near the N-terminus that is conserved in all of the vertebrate *Distal-less* family members that we have designated the '*Distal-less* domain'. Although the sequence of this region does not indicate its role, its conservation strongly argues its importance for *Dlx* function.

3.2. Expression of Dlx genes suggests participation in craniofacial patterning

Unlike previously reported murine *Dlx* genes (Price et al, 1991; Porteus et al, 1991; Robinson et al, 1991; Dollé et al, 1992; Bulfone et al, 1993; Simeone et al, 1994), *Dlx*-3 is not expressed at detectable levels in the ventral forebrain and it displays much more restricted expression in the branchial arches and in subsets of their derivatives. Comparative analysis with *Dlx*-2 expression illustrates its dynamic nature, which varies both temporally and spatially during craniofacial development. Expression of *Dlx*-2 in the branchial arches of embryos from 8.5–11.5 days p.c. has been reported before (Bulfone et al, 1993). Our study confirms and extends these results to include later stages of development and follows *Dlx*-2 expression as the various derivatives of the arches differentiate.

Dlx-2 expression starts earlier and is much more widespread than *Dlx*-3 expression in the branchial arches. *Dlx*-2 appears to be expressed in migratory neural crest cells in all of the branchial arches. In contrast, *Dlx*-3 expression is detected later, after emigration of neural crest cells has ceased, and is largely restricted to the distal tips of the first and second arch. Initially *Dlx*-3 is expressed uniformly in the distal portion of the first two arches but within a day of its activation, its expression becomes localized to the caudal portion of the first arch and the second arch. Interestingly, labeled crest cells originating from the level of rhombomere 1 and 2 in the chick displayed migration patterns very similar to the expression pattern of *Dlx*-3 in the caudal first arch (Lumsden et al, 1991), suggesting that *Dlx*-3 expression may demarcate a subpopulation of crest cells that have a precisely defined segmental origin. The expression domain of *Dlx*-2 extends more dorsally, more rostrally into the maxillary process and more caudally into all of the branchial arches. Expression of both *Dlx* genes becomes localized to distinct subsets of structures derived from the branchial arches which initially express these genes.

The differentiation of the neural crest-derived mesectoderm involved in the morphogenesis of the face relies on extensive epithelial-mesenchymal interactions (Noden, 1986; Le Douarin et al, 1993). *Dlx*-2 and -3 expression is detected in many areas where such interactions are known to occur, such as the developing teeth, whisker follicles and ear. These two genes are generally expressed in complementary and/or partially overlapping domains within these regions. *Dlx*-2 expression generally precedes that of *Dlx*-3 (for example, in the branchial arches, the teeth and whiskers), raising the possibility that *Dlx*-2 regulates *Dlx*-3 either directly or indirectly in cells that express both genes, or that *Dlx*-2 expressing cells can induce *Dlx*-3 expression in certain adjacent cells.

3.3. Comparative analysis of Dlx-3 genes in other vertebrate systems

The *Dlx*-3 related genes from mouse and other vertebrate species show extensive homology outside of the homeodomain and display common features in their developmental expression, strongly suggesting that they are true orthologs (Ekker et al, 1992; Beauchemin and Savard, 1992; Papalopulu and Kintner, 1993; Dirksen et al, 1994; Morasso et al, 1994). The murine *Dlx*-3 gene is expressed in the skin of embryos and in adults, as are other *Dlx*-3 orthologs (Beauchemin and Savard, 1992; Morasso et al, 1994; Dirksen et al, 1994). Certain *Hox* homeobox genes are also expressed in skin (Chuong et al, 1990; Bieberich et al, 1991; Detmer et al, 1993), suggesting that they may be involved in conferring spatial information to this organ. In contrast, the extensive expression of *Dlx*-3 in the skin and associated hair follicles coupled with the observation that the *Dlx*-3 gene is activated during the induced differentiation of mouse keratinocytes in vitro (Morasso et al, 1994), indicate that this gene is more likely to be involved in cellular differentiation than in providing positional information in the skin.

The frog gene *Xdll*-2 is also reported to be expressed in the branchial arches (Dirksen et al, 1994), although these authors did not assess expression during later stages of craniofacial development extensively. The zebrafish *dlx*3 gene is expressed during inner ear development (Ekker et al, 1992), as is the murine homolog. There are some differences in the expression patterns in these disparate vertebrate species that can be accounted for by species-specific variations in the ontogeny, architecture and function of the inner ear of fish and mammals. Thus, it would appear that spatially restricted *Dlx*-3 expression is involved in the regulation of inner ear development in both vertebrate species.

The newt ortholog, *NvHBox*-4, and the related gene *NvHBox*-5 are expressed in the skin and brain of adults (Beauchemin and Savant, 1993). Expression of *Dlx*-3 has not been detected in the mouse brain, suggesting that either the newt gene is not a true ortholog or that this discrepancy is due to species-related variability. The newt genes are also expressed in regeneration blastemas of the limbs and tail and, therefore, appeared to be reactivated during regeneration. Our study has focused on the expression of *Dlx*-3 in the head, although expression of *Dlx*-3 (M. Morasso and T. Sargent, personal communication), as well as *Dlx*-1 and -2 genes (Dollé et al,

1992; Bulfone et al, 1993), has been detected in the apical ectodermal ridge of mouse embryo limb buds.

3.4. The role of homeobox genes in craniofacial development

At the time of migration from the neuroepithelium, the cranial neural crest cells have acquired intrinsic patterning information concerning their position along the rostrocaudal axis and the structures they give rise to (Noden, 1983). There is increasing evidence that homeobox genes are involved in specifying this information (reviewed in McGinnis and Krumlauf, 1991). In mouse embryos, cognate members of the *Hox* gene clusters are expressed in a dynamic and coordinated fashion along the rostrocaudal axis. In the hindbrain, the rostral boundaries of *Hox* expression domains coincide with alternating rhombomere borders, and each rhombomere is characterized by a distinct *Hox* expression profile that results from variations in both the duration and levels of *Hox* expression. This expression profile, or '*Hox* code', is believed to impart positional identity along the anteroposterior axis. With the exception of *Hoxa*-2 (Lumsden and Prince, 1994), the neural crest cells emigrating from the rhombomeres express the

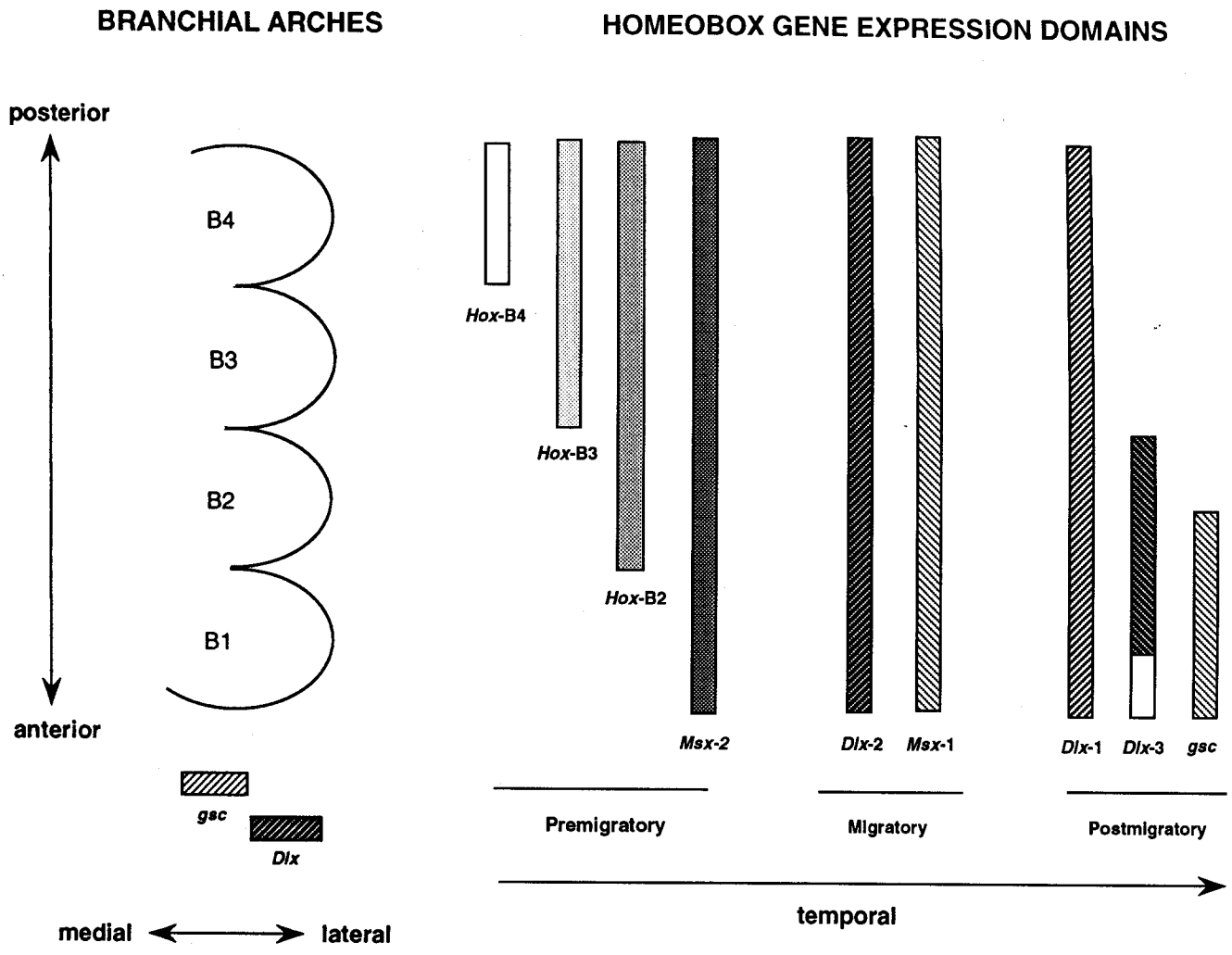

Fig. 9. Homeobox gene expression domains in the branchial arches. The temporal and spatial patterns of expression suggest that the neural crest cells that give rise to the arches express distinct subsets of homeobox genes before, during and after emigration. The boxed regions indicate the temporal and spatial expression domains of *Dlx*-1, -2 and -3 and several other representative homeobox genes, *Hox*, *Msx* and *gsc* (see text). For simplicity, only the *Hox*-B genes are diagramed, though other *Hox* family members are expressed in these regions as well (McGinnis and Krumlauf, 1992). The location of the boxed domains relative to the diagram of the branchial arches (B1–4) indicates their anteroposterior and mediolateral positions. The open boxed area for *Dlx*-3 shows that its rostral boundary initially extends to the entire first arch but eventually regresses to the posterior half of this arch. Based on published observations of the location and initial appearance of detectable expression, the neural crest cells expressing individual homoebox genes are classified as premigratory, migratory or postmigratory. Assignment to these classes is not absolute, since the genes have not all been directly compared by the same investigator and since levels of expression below the limit of detection would not have been reported. Variation along the dorsoventral axis is not represented here.

same set of *Hox* genes as the neuroectoderm at their point of origin.

We have shown that the *Dlx*-2 and -3 genes are also expressed in overlapping, but distinct, regions of the cranial neural crest that give rise to the branchial arches. *Dlx*-1 is also differentially expressed in the branchial arches (Dollé et al, 1992). Its early expression pattern in the mesenchyme of all of the arches is very similar to that of *Dlx*-2, although transcripts of *Dlx*-1 are initially detected later in development (10.5 days p.c.) (Dollé et al, 1992). The *Distal-less* expression domains partially overlap with those of the *Hox* genes along the rostrocaudal axis, but extend more rostrally into the first arch, where *Hox* gene expression has not been detected.

Several other homeobox genes that are not part of the *Hox* gene family are also expressed in the cranial neural crest and branchial arches at this stage of embryogenesis. The expression domains of these genes (diagrammed in Fig. 9) vary temporally and spatially along the dorsoventral and mediolateral as well as the rostrocaudal axes. The *Msx* genes are rather broadly expressed in cranial neural crest at or before the time of emigration (Hill et al, 1989; Robert et al, 1989; MacKenzie et al, 1991a,b; Graham et al, 1993). Transcripts become localized to the nasal processes and the branchial arch mesenchyme and later become prominently expressed in the developing teeth and other sites of epithelial-mesenchymal interaction (MacKenzie et al, 1992). As their expression patterns suggest, these genes play important roles in the morphogenesis of the head and teeth. Mutations in the *Msx*-2 gene give rise to anomalies in cranial development in humans (Jabs et al, 1993), and loss of *Msx*-1 gene function in mice results in craniofacial abnormalities and arrested tooth development (Satokata and Maas, 1994).

The mouse *goosecoid* gene is expressed in postmigratory neural crest cells of the first and rostral portion of the second arch (Gaunt et al, 1993). The expression domain of *goosecoid* in this area is complementary and exclusive to that of *Dlx*-1, being restricted to the medial parts of the first arch, whereas *Dlx*-1 (Dollé et al, 1992), *Dlx*-2 (Bulfone et al, 1993; this report) and *Dlx*-3 (this report) are expressed in the lateral aspects of the arch. *Goosecoid* is also expressed in the first branchial cleft and around the nasal pits, and thus seems to overlap with the *Dlx*-3 expression domain in this region both temporally and spatially. In contrast, *goosecoid* expression is restricted to the ventral portion of the otic vesicle and the cochlear duct that derives from it, while both *Dlx*-2 and -3 are expressed exclusively in derivatives of the dorsal otic vesicle, the utricle and semicircular canals.

The differential expression of the *Dlx* genes with these and other transcription factor genes in the branchial arches strongly suggests that these genes participate in craniofacial development through unique functions and/or through combinatorial interactions. The *Dlx* genes could function in specifying or in elaborating spatial information as appears to be the case for the *Hox* genes, or they could function, as has been postulated for the *Msx* genes, as mediators in the signalling processes that take place during tissue interactions (Mackenzie et al, 1991a,b; Takahashi et al, 1991; Brown et al, 1993).

Temporal differences in expression of homeobox genes in the cranial neural crest suggest potential regulatory relationships. *Hox* and *Msx*-2 gene expression is detected in the hindbrain and associated neural crest before emigration. *Dlx*-2 and *Msx*-1 expression is detected in the crest cells slightly later, as the cells start to migrate, and *Dlx*-3 and *goosecoid* transcripts are apparently expressed after the cells have reached the distal aspects of the arches. Perhaps *Hox* genes, which are postulated to impart positional address, modulate the expression of the *Dlx* genes. In this regard, it would be interesting to examine whether *Dlx* gene expression is altered in null mutants of *Hox* genes that are normally expressed in the arches (Chisaka and Capecchi, 1991; Lufkin et al, 1991; Chisaka et al, 1992, Gendron-Maguire et al, 1993; Rijli et al, 1993). Intriguingly, the *Dll* gene is subordinate to homeotic genes in *Drosophila* (Vachon et al, 1992; O'Hara et al, 1993). Expression of *Dll* in the head is required for the proper specification of the ventral maxilla and is regulated by the *Deformed* gene (O'Hara et al, 1993). Thus, in *Drosophila*, *Dll* functions as a mediator of homeotic gene activity by refining the spatial patterns established by the homeotic genes, a situation that may be recapitulated in the mouse during the morphogenesis of the head.

4. Experimental procedures

4.1. Animals

To obtain embryos, female FVB/N mice were superovulated and mated. Noon of the plug day was counted as 0.5 days post coitum (p.c).

4.2. Library screening and isolation of clones

Several embryonic cDNA λgt 10 libraries were screened with a mixture of amplified mouse *Distal-less* homeobox sequences as described (Robinson et al, 1991). DNA probes were labeled with ^{32}P by the random-primed method (Feinberg and Vogelstein, 1983). Two partial clones were isolated from an 8.5-day (Fahrner et al, 1987) and a 12.5-day (Joyner and Martin, 1987) cDNA library. The inserts were subcloned into Bluescript and sequenced using the dideoxy chain termination method (Sanger et al, 1977). The longer of these cDNA sequences was used to screen a genomic DNA library and one clone was isolated. It was subcloned according to standard methods (Sambrook et al, 1989) and partially sequenced. The sequences were analyzed using GCG programs (Devereux et al, 1984).

4.3. Northern blot hybridization

RNA was prepared with RNazol (Cinna-Biotex) according to manufacturer and was separated on 1.2% formaldehyde-agarose gels, transferred to nylon membranes (Stratagene) and hybridized with random primed, [^{32}P]-labeled probes in Rapid-hyb buffer (Amersham) at 65°C. Stringent washes were performed at 65°C in 0.1 × SSC and 0.1% SDS. Probes were generated from restriction fragments and included the 1.1-kb insert from the longest *Dlx*-3 cDNA clone and the rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH).

4.4. In situ hybridization

Hybridization to sections. Embryos were fixed in 4% paraformaldehyde in PBS, embedded in paraffin and 3.5 μm sections were mounted on poly-lysine coated slides. Sense and antisense probes were generated from appropriately linearized plasmids by in vitro transcription with T3 and T7 polymerase (Stratagene) and ^{35}S-UTP (Amersham). The plasmid for *Dlx*-3 contained a 1.1-kb cDNA insert. Probes for *Dlx*-2 were as described previously (Robinson et al, 1991). Hybridization was performed as previously described (Mackem and Mahon, 1991). The slides were coated with Kodak NTB-2 emulsion and developed after 10–14 days exposure at 4°C.

Whole mount hybridization. The procedure described by Conlon and Rossant (1992) was used. Embryos were fixed in 4% paraformaldehyde in PBS (phosphate buffered saline) on ice for 2 h, washed in PBT (PBS containing 0.1% Tween 20) and dehydrated in methanol. They were bleached, rehydrated and treated with Proteinase K as described. After fixing in 4% paraformaldehyde and 0.2% glutaraldehyde, they were treated with 0.1% sodium borohydride. Prehybridzation was carried out for 1 h at 63°C in hybridization buffer (50% formamide, 0.75 M NaCl, 10 mM PIPES pH 6.8, 1 mM EDTA, 100 μg/ml *E. coli* tRNA, 0.1% BSA, 1% SDS). Digoxigenin-labeled riboprobes, prepared with a Genius kit (Boehringer Mannheim), were used for hybridization overnight at 63°C. The embryos were washed as described and subjected to immunohistochemical detection of the hybridized probes. A color reaction was usually seen after 1–2 h. In some instances, strongly stained specimens were dehydrated and embedded in paraffin and thin sections were prepared to determine the tissue distribution of labeled cells.

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Note added in proof

The sequence of *Dlx*-3 is identical to the partial sequence of *Dlx*-7 reported in Weiss et al., J. Exp. Zool., in press.

References

Asano, M., Emori, Y., Saigo, K. and Shiokawa, K. (1992) J. Biol. Chem. 267, 5044–5047.
Balling, R., Mutter, G., Gruss, P. and Kessel, M. (1989) Cell 58, 337–347.
Beauchemin, M. and Savard, P. (1992) Dev. Biol. 154, 55–65.
Bieberich, C.J., Ruddle, F. and Stenn, K.S. (1991) Ann. New York Acad. Sci. 642, 462–464.
Brown, J.M., Wedden, S.E., Millburn, G.H., Robson, L.G., Hill, R.E., Davidson, D.R. and Tickle, C. (1993) Development 119, 41–48.
Bulfone, A., Kim, H.-J., Puelles, L., Porteus, M.H., Grippo, J.F. and Rubenstein, J.L.R. (1993) Mech. Dev. 40, 129–140.
Chisaka, O. and Capecchi, M.R. (1991) Nature 350, 473–479.
Chisaka, O., Musci, T.S. and Capecchi. M.R. (1992) Nature 355, 516–520.
Chuong, C.-M., Oliver, G., Ting, S.A., Jegalian, B.G., Chen, H.M. and De Robertis, E.M. (1990) Development 110, 1021–1030.
Cohen, S.M. and Jürgens, G. (1989) EMBO J. 8, 2045–2055.
Conlon, R.A. and Rossant, J. (1992) Development 116, 357–368.
Detmer, K., Lawrence, J. and Largman, C. (1993) J. Invest. Derm. 101, 517–522.
Devereux, J., Haeberli, P. and Smithies, O. (1984) Nuc. Acids Res. 12, 387–395.
Dirksen, M.-L., Mathers, P. and Jamrich, M. (1993) Mech. Dev. 41, 121–128.
Dirksen, M.-L., Morasso, M., Sargent, T. and Jamrich, M. (1994) Mech. Dev. 46, 63–70.
Dollé, P., Price, M. and Duboule, D. (1992) Differentiation, 49, 93–99.
Ekker, M., Akimenko, M.-A., Bremiller, R. and Westerfield, M. (1992) Neuron 9, 27–35.
Fahrner, K., Hogan, B.L.M., Flavell, R.A. (1987) EMBO J. 6, 1265–1271.
Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.
Gaunt, S.J., Blum, M. and De Robertis, E.M. (1993) Development 117, 769–778.
Gendron-Maguire, M., Mallo, M., Zhang, M. and Gridley, T. (1993) Cell 75, 1317–1331.
Graham, A., Heyman, I. and Lumsden, A. (1993) Development 119, 233–245.
Guthrie, S., Muchamore, I., Kuroiwa, A., Marshall, H., Krumlauf, R. and Lumsden, A. (1992) Nature 356, 157–159.
Hardy, M.H. (1992) Trends Genet. 8, 55–61.
Hill, R.E., Jones, P.F., Rees, A.R., Sime, C.M., Justice, M.J., Copeland, N., Jenkins, N.A., Graham. E. and Davidson, D.R. (1989) Genes Dev. 3, 26–37.
Jabs, E.W., Müller, U., Li, X., Ma, L., Luo, W., Haworth, I.S., Klisak, I., Sparkes, R., Warman, M.L., Mulliken, J.B., Snead, M.L. and Maxson, R. (1993) Cell, 75, 443–450.
Joyner, A. and Martin, G. (1987) Genes Dev. 1, 29–38.
Kessel, M. and Gruss, P. (1991) Cell 67, 89–104.
Kozak, M. (1989) J. Cell Biol. 108, 229–241.
Krumlauf, R. (1992) BioEssays 14, 245–252.
Krumlauf, R. (1993) Trends Genet., 9, 106–112.
Kuratani, S.C. and Eichele, G. (1993) Development 117, 105–117.
Le Douarin, N.M., Ziller, C. and Couly, G.F. (1993) Dev. Biol. 159, 24–49.

Le Lièvre, C. and Le Douarin, N. (1975) J. Embryol. exp. Morphol. 34, 125–134.
Lufkin, T., Dierich, A., LeMeur, M., Mark, M. and Chambon, P. (1991) Cell 66, 1105–1119.
Lumsden, A. (1988) Development 103 Suppl. 155–169.
Lumsden, A. Spawson, N. and Graham, A. (1991) Development 113, 1281–1291.
Mackem, S. and Mahon, K.A. (1991) Development 112, 791–806.
MacKenzie, A., Leeming, G., Jowett, A.K., Ferguson, M.W.J. and Sharpe, P.T. (1991a) Development, 111, 269–285.
MacKenzie, A., Ferguson, M.W.J. and Sharpe, P.T. (1991b) Development 113, 601–611.
MacKenzie, A., Ferguson, M.W.J. and Sharpe, P.T. (1992) Development 115, 403–420.
McGinnis, W. and Krumlauf, R. (1992) Cell 68, 283–302.
Martin, P. and Swanson, G.J. (1993) Dev. Biol. 159, 549–558.
Morasso, M., Jamrich, M. and Sargent, T. (1994) Dev. Biol. 162, 267–276.
Noden, D.M. (1973) In Bosma, J. (ed.), Fourth Symposium on Oral Sensation and Perception: Development in the Fetus and Adult, DHEW Pub. No. (NIH) 73–546. pp. 9–33.
Noden, D.M. (1975) Dev. Biol. 42, 106–130.
Noden, D.M. (1983) Dev. Biol. 96, 144–165.
Noden, D.M. (1986) J. Craniofacial Genet. Dev. Biol., Suppl. 2, 15–31.
O'Hara, E., Cohen, B., Cohen, S.M. and McGinnis, W. (1993) Development 117, 847–856.
Papalopulu, N. and Kintner, C. (1993) Development 117, 961–975.
Porteus, M.H., Bulfone, A., Ciaranello, R.D. and Rubenstein, J.L.R. (1991) Neuron 7, 221–229.
Price, M., Lemaistre, M., Pischetola, M., DiLauro, R. and Duboule, D. (1991) Nature 351, 748–751.
Prince, V. and Lumsden, A. (1994) Development 120, 911–923.
Rijli,F.M., Mark, M., Lakkarju, S., Dierich, A., Dollé, P. and Chambon, P. (1993) Cell 75, 1333–1349.
Robert, B., Sassoon, D., Jacq, B., Gehring, W. and Buckingham, M. (1989) EMBO J. 8, 91–100.
Robinson, G.W., Wray, S. and Mahon, K.A. (1991) The New Biol. 3, 1183–1194.
Sadler, T.W. (1990) Langman's Medical Embryology, 6th ed. Williams and Wilkins, Baltimore.
Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
Satokata, I. and Maas, R. (1994) Nat. New Genet. 6, 348–355.
Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Nat. Acad. Sci. USA 74, 5463–5467.
Sher, A.E. (1971) Acta Otolaryng. Suppl. 285, 1–77
Sechrist, J., Serbedzija, G.N., Scherson, T., Fraser, S.E. and Bronner-Fraser, M. (1993) Development 118, 691–703.
Selski, D.J., Thomas, N.E., Coleman, P.D. and Rogers, K.E. (1993) Gene 132, 301–303.
Serbedzija, G.N., Bronner-Fraser, M. and Fraser, S.E. (1992) Development 116, 297–307.
Simeone, A., Acampora, D., Pannese, M., D'Esposito, M., Stornaiuolo, A., Gulisano, M., Mallamaci, A., Kastury, K., Druck, T., Huebner, K. and Boncinelli, E. (1994) Proc. Nat. Acad. Sci. USA 91, 2250–2254.
Sunkel, C.E. and Whittle, J.R.S. (1987) Roux's Arch. Dev. Biol. 196, 124–132.
Takahashi, Y. and Le Douarin, N. (1990) Proc. Nat. Acad. Sci. USA 87, 7482–7486.
Takahashi, Y., Bontoux, M. and Le Douarin, N. M. (1991) EMBO J. 10, 2387–2393.
Vachon, G., Cohen, B., Pfeifle, C., McGuffin, M.E., Botas, J., and Cohen, S.M. (1992) Cell 71, 437–450.